IMPACT OF PLANT GROWTH PROMOTING PROPERTIES OF *Lactobacillus plantarum* AND ANTIBIOTIC FORMULATIONS

KEERTHIKA R

(20PBT009)

M.Sc in Biotechnology

A Thesis submitted to

AVINASHILINGAM INSTITUTE FOR HOME SCIENCE AND HIGHER EDUCATION FOR WOMEN,

COIMBATORE–641 043.

In partial Fulfillment of the Requirement for the Degree of

Master of Science in Biotechnology

May, 2022

Certificate

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Signature of the Supervisor

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Introduction

1. INTRODUCTION

Fungi are the most common plant pathogens, and they cause a wide spectrum of significant plant diseases. Infected seeds, soils, agricultural debris, crops and weeds have been involved in the spreading of fungal infections in plants (De Silva *et al*., 2016; Konopka *et al*., 2019). Plant fungal infections are responsible for the majority of crop loss in agriculture. Collectively, the phytopathogens have developed unique mechanisms to attack plants, there by invading into the plant systems and utilizing nutrients forcefully for their growth and development. These infections have the ability to replicate asexually andsexually, can overcome plant immune defense mechanisms. Ultimately, it has a negative impact on plant health, homeostasis, and physiology (Matrose *et al*., 2021).

Spores of the phytopathogens have been easily germinated underfavorable conditions including humidity,low molecular mass nutrients and a suitable host. Self-inhibitors can keep fungal spores alive for several years by preventing the process of germination until favorable conditions (Comt *et al*., 2015). Spores could be easily spread to the infected areas in plants by wind, birds, humans, insects, water and infected plant parts. Fungi are responsible for more than 80% of plant diseases. Approximately 8000 fungal species cause nearly 100,000 diseases in plants (Raut and Karuppayil, 2014).

Phytopathogenic fungal strains have caused a specific disease named as blight diseases especially in potatoes, cereal, wheat, brown spot of rice, coffee rust, sigatoka disease of banana, chestnut blight, downy of grape, wheat stem rust and rubber leaf blight. These diseases could ultimately have a negative impact on agricultural output and is also endanger human health by reducing the quality and quantity of food products. As a result, these phytopathogens generate major issues for farmers, policymakers and consumers (Shuping., 2016).

Plant pathogens such as *magnaporthe oryzae* and *Colletotrichum spp*. cause devastating infections. *Blumeria graminis* affects crop yield and *Fusarium graminearum* reduces the crop quality. *Mycosphaerella graminicola* can mutate and infect many plants in the same area, reducing the ability of plants to resist pathogenic invasion. *Botrytis cinerea* has 200 plant hosts and *F*. *oxysporum* has roughly 100 plant hosts, therefore one species can cause several diseases in a wide range of plants. A single pathogen named as *M. oryzae* can cause significant grain loss (Martinez *et al*., 2016).

Pisum sativum is a pea plant, fabaceae family which is known to produce pods and have high concentration of vitamins, minerals, antioxidants and phytonutrients. Peas are the second greatest protein source for the human but, the pathogen attack and climate change has drastically reduced the yield of pea. Aphanomyces root rot is one of the fungal infection in pea plants that causes yellowness of leaves. This begin at the bottom of the pea plant and progress higher, reduced the pod production, soft dark lesions on roots and stunted growth of plants. *Mycosphaerella pinodes* illness appears on leaves as little purple flecks that progress to a dark brown lesion that might cause the leaves to dry up. The seeds that have been infected with pathogens showed shrinkage and dark brown coloration, which results in girdled stems, stunted growth or plant death (Sulima *et al*., 2022). *Rhizopus* spp. have been associated with pea seeds and known to produce toxic metabolites which severely affect the seed germination rate in pea plants.

Rhizopus species have reported with several storage rot root diseases in many plants such as leak disease in strawberries and tomatoes, soft rot and ring rot in sweet potatoes, pole rot in tobacco leaves, and fruit rot in papayas and stone fruits. In agriculture bulk of postharvest diseases are caused by more than 100 species of fungi, and postharvest infections can destroy 10% to 30% of crop production. Perishable commodities can lose up to 50% of their value in poor countries and tropical locations (Kim *et al.*, 2016). Plant fungal pathogens can attack and enter into the host in a numerous way, some pathogens enter their host through mechanical and chemical pressure, whereas others enter through wounds and stomata (Montesinos *et al*., 2017).

Since, plant pathogenic fungi are a major causative agent for 20-40% plant diseases. To overcome the crop loss issues faced by the farmers, chemical fungicides are the common way to prevent the fungal infections in plants. Fungicides are used to control the excessive loss in crop yield and quality. Indiscriminately, chemical fungicides are effective in managing fungal infections in plants, but it has reported with several limitations. Furthermore, the extensive usage of chemical pesticides and fertilizers leads to deadly medical complications in human including, cancer (Chen *et al*., 2020).

Various agrochemical agents have been developed and used to fight against plant fungal pathogens to mitigate the loss of agricultural crops. Some of these agrochemicals are toxic to humans.Many of these chemical pesticides have a negative environmental effects for soil organisms, insects, and plant pollinators. By concerning the harmful effects of the

chemical agro pesticides, farmers are paying more attention to pesticides developed from natural compounds (Eloff and Gaw, 2016). Effective biological products that are affordable, less harmful, and effective and using plant-based products are of great interest (Eloff and Gaw, 2016).

Biological agents have been developed from bacteria or fungi has significant applications in the control of plat-parasitic fungi. Although, few commercial formulations have been developed for the effective control of pests, their usage is limited due to their toxicological nature. The major anticipated discovery in the field of agriculture is to develop antifungal compounds from natural sources for the prevention and management of crop loss. To mitigate the risk of crop loss and enhance the food safety, new and natural fungicides need to be discovered. This lead to the development of novel fungicides and fertilizers from microorganisms, especially the metabolites from bacteria is increasing at global level (Somashekaraiah *et al*., 2021; Li *et al*., 2020).

Among several beneficial bacteria, the metabolites from lactic acid bacteria (LAB) showed greatest antifungal spectrum against wide range of phytopathogens (Montesinos *et al*., 2017). *Lactobacillus plantarum* have been recorded with antifungal activities against several phytopathogens including, *Fusarium* species, aflatoxin producing *Aspergillus parasiticus* and several dairy pathogens (Tsuda *et al*., 2016). *Lactobacillus plantarum* can used as a biological control agent to prevent bacterial and fungal crop diseases. In addition to this, it can be used to boost the plant growth and defend against disease-causing pathogens (Stefanovic *et al.,* 2017).

Panchagavyam is a term used to describe five important substances from cow such as milk, urine, ghee, curd and dung. All these products have important medicinal properties and recorded with plant growth promoting and antagonistic activities against several plant pathogens (Bajaj *et al*., 2022). Panchagavyam promotes soil fertility by enhancing the organic matter, macro and micronutrient levels thus, increase the nutrient uptake in plants. It is known to encourage the growth of beneficial microbes in soil, reproduction and preserving good soil health. It also improves soil physical qualities by reinforcing the porosity, balancing aggregate stability, managing soil pH and nutrient profile. Panchagavyam in agricultural fields has a significant impact on crop growth and productivity by increasing beneficial soil bacteria surrounding the roots (Ram *et al.,* 2017; Praburaman *et al*., 2020). Panchagavya ensures that no toxic components in their formulations to act as fertilizers,

herbicides, insecticides, or antibiotics and less cost-effective. As an organic fertilizer, it is evident that, it could boost the soil fertility, improve earthworm quality and promote crop health (Christophe *et al*., 2019).

Bio-organic fertilizers maintain the soil quality, increase the earthworm populations, fertility of the soil and improvement in the nitrification process (Kumar *et al*., 2020; Praburaman *et al*., 2020).

Based on this background, the present study entitled **"Impact of plant growth promoting properties of *Lactobacillus plantarum* and antibiotic formulations"** is formulated with the following objectives:

- To develop the bioformulations using metabolites of *Lactobacillus plantarum* and panchgavyam for sustainable agriculture
- To analyze the *in vitro* plant growth promoting properties of the biofomulations
- To determine the antifungal potential of developed formulations on fungal isolates from *Pisum sativum*
- To validate the efficiency of the developed formulations on pot plants of peas

Review of Literature

2. REVIEW OF LITERATURE

Plant fungal pathogens play a crucial role in the profitability, quality and quantity of plant production. These phytopathogens are persistent in avoiding plant defences causing diseases and quality losses around the world that amount to billions of US dollars annually. To control the scourge of plant fungal diseases, farmers have used fungicides to manage the damage of plant pathogenic fungi. Drawbacks such as development of resistance and environmental toxicity associated with these chemicals have motivated researchers and cultivators to investigate other possibilities. Microbial based biocontrol agents have been applied to the crops for the enhancement of plant growth promoting characteristics and also act as biocontrol agent to inhibit the growth of fungal pathogens.

A brief review of literature on "**IMPACT OF PLANT GROWTH PROMOTING PROPERTIES OF *Lactobacillus plantarum* AND ANTIBIOTIC FORMULATIONS**" is discussed under following headings:

2.1. Phytopathogens

2.2. Fungal diseases on crops

2.3. *Rhizopus* as plant pathogen

2.4. Pea plants

2.5. Biocontrol agents

2.5.1. *Lactobacillus Plantarum*

2.5.2. panchagavya

2.6. Plant growth regulators

2.1. Phytopathogens

Plant fungal pathogens cause most of the diseases occurring in agricultural and horticultural setups Collectively, the phytopathogens have developed mechanisms and ways to attack any plant, seeking entry and sourcing nutrients forcefully for growth and development, for plant fungal diseases to occur the plant fungal pathogen must be able to germinate on the surface of a suitable host. The plant fungal pathogen spores can only germinate when conditions are favourable. This includes suitable humidity by rain or dew, availability of low molecular mass nutrients and a suitable host. Fungal spores can remain viable for many years using self-inhibitors to stop germination until favourable conditions are present. When the conditions are favourable the plant fungal pathogens form infection structures like the aspersorium and the infection peg, for the hyphae to penetrate the host. Pathogens like *Colletotrichum gloeosporioides* that cause diseases in avocados, can use their host waxes to infect their host (Jain *et al.,* 2019).

Fungal diseases may be minimized by the reduction of the inoculums, inhibition of its virulence mechanisms and promotion of genetic diversity in the crop. The use of chemical fungicides in agriculture has been proven to bring about various benefit such as reducing the fungal infection that may rob water and nutrients from crop plants or may cause spoilage while the products are transported to the market. Fungicides may also prevent the growth of fungi that produce toxins, such as aflatoxins. Worldwide, 5.7 billion pounds of pesticides were used, of which 0.5 billion were fungicides. There are numerous classes of fungicides, with different modes of action as well as different potential for adverse effect on health and environment. Indicated 311 compounds are registered and used as fungicides to control various plant fungal diseases of these, seven agents are antagonistic microorganisms and only one agent is derived from plant extract, i.e., extract of *Reynoutria sachalinensis* (Aboody *et al*., 2020).

Most fungicides can cause acute toxicity, and some cause chronic toxicity as well. The use of chemical pesticides has been known to cause various environmental and health problems. The International labor organization (ILO) estimates that as much as 14 per cent of all occupational injuries are due to exposure to pesticides and other agrochemical constituents. The World Health Organization (WHO) and the united nations environment programme estimates that each year, three million workers in agriculture in developing world experience severe poisoning from pesticides, about 18,000 of whom die appropriate

technological improvement, which results in more effective use of natural resources is required in agriculture. One of them is the use of microbial antagonists (Luchi *et al*., 2020).

Many microbial antagonists have been reported to possess antagonistic activities against plant fungal pathogens, such as *Pseudomonas fluorescens, Agrobacterium radiobacter, Bacillus subtilis, B. cereus, B. amyloliquefaciens, Trichoderma virens, Burkholderia cepacia, Saccharomyces* sp, *Gliocadium* sp. The successful control by these antagonists mainly against the diseases caused by following genera of pathogens, *Alternaria, Pythium, Aspergillus, Fusarium, Rhizoctonia, Phytophthora, Botrytis, Pyricularia* and *Gaeumannomyces*. This paper describes briefly the biocontrol potential of microbial antagonists particularly against plant fungal pathogens (Jain *et al.*, 2019).

2.2 Fungal Diseases on Agricultural Crops

More than 10,000 species of fungi, can cause disease in plants .Classes of fungi that commonly cause diseases in agricultural crops are *Plasmodiophoromycetes* (cause clubroot of crucifers, root disease of cereals, and powdery scab of potato), *Oomycetes* (cause seedling damping-off, late blight, downy mildews, and white rust disease), *Zygomycetes* (cause soft rot of fruit*), ascomycetes* and *deuteromycetes*cause leaf spots, blights, cankers, fruit spots, fruit rots, anthracnoses, stem rots, root rots, vascular wilts, soft rot (Borgatta *et al.*, 2018).

Apple scab, caused by the fungus *Venturia inaequalis* is the most important diseases of apples worldwide, and it very likely occurs in every country where apples are grown. In some circumstances, the losses from apple scab can be 70% or more of the total fruit value *Macrophomina phaseolina*, is important root pathogen and causes dry root rot or stem canker, stalk rot or charcoal rot of over 500 plant species. The genus *Fusarium* contains a number of species, which have been recognized for a long time as being important plant pathogens. *Rhizoctonia solani* exists in the soil and attacks more than 2000 species of plants (Petrasch *et al*.,2019).

They penetrate plants by stomata and wounds caused by pruning, harvesting, hail, insects, various illnesses, and mechanical damage (Borgatta *et al*.,2018).

- ✓ Target spot – *Alternaria solani* (tomatoes)
- ✓ Aphanomyces root rot – *Aphanomyces euteiches pv. phaseoli* (beans)
- ✓ Aschochyta collar rot (peas)

- ✓ Gummy stem blight – *Didymella bryoniae* (cucurbits)
- ✓ Alternaria leaf spot – *Alternaria cucumerina* and *A. alternata* (cucurbits)
- ✓ Black leg – *Leptosphaeria maculans* (brassicas)
- ✓ Ring spot – *Mycosphaerella brassicicola* (brassicas)
- ✓ Late blight – *Septoria apiicola* (celery)
- ✓ Cercospora leaf spot – *Cercospora beticola* (beets)
- ✓ Leaf blight – *Septoria petroelini* (parsley)
- ✓ Septoria spot – *Septoria lactucae* (lettuce)
- ✓ Leaf blight – *Stemphylium vesicarium* (spring onions)
- ✓ Leaf blight – *Alternaria dauci* (carrots).

Foliar diseases are caused by fungus. Downy mildews, powdery mildews, and white blister are three of the most common foliar diseases. Other fungi that cause soilborne illnesses include clubroot, *pythium species, fusarium species, rhizoctonia species, sclerotinia and sclerotium* species (Fisher *et al*., 2020).

Table 1: Fungal diseases in major crops

Fungal disease	**Factors conducive to spread**	**Crops affected**	**Symptoms**
White blister/White rust (*Albugo candida*)	Optimum conditions for disease development are 3-4 hours in mild temperatures (6-24°C).	Brassicas (including Asian leafy brassicas).	White blisters and swellings on leaves and heads of affected plants; blisters consist of masses of white dust-like spores; up to 100% losses have been

			reported.
Downy mildews (individual species damage particular crop families)	High humidity, leaf wetness and cool to mild temperatures (10-16 °C).	Wide host range including onions; peas; lettuce; celery; spinach; kale; herbs; cucurbits; brassicas; Asian leafy brassicas.	Symptoms usually begin with yellowish leaf spots which then turn brown; downy growth appears on underside of leaves.
Powdery mildews (some species are restricted to particular crops or crop families)	Moderate temperatures (20-25°C); relatively dry conditions (unlike downy mildews).	Wide host range and very common, especially in greenhouse crops: cucumber; melons; pumpkin; zucchini;parsnip; beetroot; potato; herbs; peas; bitter melon;tomato; capsicum; Brussels sprouts; cabbage; swedes.	Small, white, powdery patches on most above-ground surfaces; usually observed first on undersides of leaves but eventually cover both surfaces; affected leaves become yellow, then brown and papery and die.
Clubroot (*Plasmodiophora brassicae*)	Warm weather; acidic soil (pH less than 7); high soil moisture.	Brassicas (including Asian leafy brassicas).	Plants are yellow and stunted and may wilt in hotter parts of the day; large malformed ‘clubbed’ roots

			which prevent the uptake of water and nutrients, reducing the potential yield of the crop.
Pythium species	Cold, wet soil conditions; known as water moulds, they enter untreated water supplies; water supplies for irrigation and hydroponics should be tested regularly.	Many vegetable crops in including cucurbits; brassicas; lettuce.	May kill seedlings, which die before they emerge or soon after emergence; plant collapse.

2.3. *Rhizopus* as plant pathogen

Over the past several years, the incidence of invasive fungal infections affecting human health has dramatically increased. While the introduction of new antifungal agents in the past decade has removed the issue of toxicity as the major factor in treatment, application of many newer antifungi still suffers from significant drawbacks. On the other hand, in agriculture the widespread use of fungicides during crop management has gained major attention owing to the increased costs, handling hazards, concern about food contamination, biomagnification, and overall threat to human health and the environment. Thus there is a need for improved antifungal and safer fungicides (Pang *et al.,* 2021).

Fungal pathogens affect a wide range of hosts and it is not unusual that a single species causes both human infections and crop loss. Examples of such species are *Rhizopus*

stolonifer and *Aspergillus niger*. *A. niger* is an opportunistic human pathogen causing *aspergillosis* and is commonly responsible for *otomycosis.* It is also the causative organism for spoilage of many freshly harvested fruits and vegetables, in particular grapes, onions, and peanuts. Infected areas are characterized by moldy patches with heavy black sporulation, resulting in the common name for *A. niger*, 'black mold.' Under the right conditions, *A. niger* infections in plants can be systemic. Unlike *A. niger, R. stolonife*r is a saprobic fungus. It is commonly found throughout the environment (Kong *et al*., 2019)

In humans, *R. stolonifer* causes *zygomycosis*, which includes pulmonary, gastrointestinal, rhinocerebral, genitourinary, mucocutaneous and disseminated infections. Alarmingly, an increasing numbers of cases with *R. stolonifer* acting as an opportunistic pathogen have been reported. *R. stolonifer* is known to cause soft rot in many agriculturally crop varieties including strawberries, tomatoes, papayas, and sweet potatoes (Zhang *et al*., 2020).

Kingdom: **Fungi**
Phylum: **Zygomycota**
Order: **Mucorales**
Family: **Mucoraceae**
Genus: ***Rhizopus***

The genus *Rhizopus* contains several species, *Rhizopus arrhizus, Rhizopus azygosporus, Rhizopus microsporus, Rhizopus schipperae,* and *Rhizopus stolonifer* are the most common. *Rhizopus species* can be distinguished by morphological characteristics such as the length of rhizooids and sporangiophores, the diameter of sporangia, the shape of columellae,and the size, shape, and surface texture of sporangiospores. The maximum growth temperature varies from species to species (Lin *et al*., 2019).

Being constantly exposed to fungal spores, many plant species have developed mechanisms protecting them from fungal infections. They produce bioactive components that are toxic to a wide variety of fungal pathogens. These natural product antifungal have shown low mammalian toxicity, are usually biodegradable, and safe for the environment. Thus plant extracts are untapped sources of potential antifungal whose antimicrobial activity has yet to be determined (Silva *et al.*, 2016).

2.4. Pea plants

Pisum sativum, in the fabaceae family that is farmed for its edible seeds and seedpods. Pea plants can be bushy or climbing, with slender stems that use tendrils to connect to a substrate. Each leaf has one to three pairs of oval leaflets and can grow to be 1–6 cm long. The plant produces white, red or purple flowers and swollen or compressed green seedpods which can be straight or curved. The pods can be anywhere between 4 and 15 centimetres long and 1.5–2.5 centimetres wide, the pea plant is an annual plant, surviving only one growing season and can reach 30–150 cm in height (Martino *et al.,* 2016).

Figure 1: *Pisum sativum*

(i) Aphanomyces root rot (Common root rot) aphanomyces euteiches

Symptoms: Include yellowing leaves that begin at the bottom of the plant and progress higher, reduced pod production,soft dark lesions on roots and severely stunted plants.

(ii) Mycosphaerella pinodes (Anamorph: Ascochyta pinodes)

Symptoms:

Symptoms may appear at any time after plant emergence. The illness appears on leaves as little purple flecks that progress to a dark brown lesion that might cause the leaves to dry up. Lesions on stems are longer than those on leaves, and they may girdle the stems. Later in the season, pods may be impacted, infecting the seeds. Seeds that have been infected may show no symptoms or show shrinkage and dark brown discolouration. Foot rot is caused by infected seeds, which results in girdled stems, stunted plants, or plant death (Martino *et al.,* 2016).

2.5. Biocontrol agents

Biological control of soil borne pathogens by introduced microorganisms has been studied over 80 years, but most of the time it has not been considered commercially feasible. However, interest and research in this area increased steadily. There is a shift toward the important role of biological control in agriculture in the future. Several companies now have programs to develop biocontrol agents as commercial products

2.5.1. *Lactobacillus plantarum*

Lactobacillus is a beneficial bacterium that helps sterilize soil and remove by products that can build up and create a harmful environment. The presence of *lactobacillus* limits the undesirable organisms in the soil. This creates a more balanced environment that is able to support plant life. Lactobacillus contributes to decomposition and disease suppression. The bacterial cycle is responsible for regulating the balance of composition in soil (chen *et al*., 2020).

Lactobacillus comes in a wide variety of species. These bacteria are "friendly" bacteria that live in our digestive, urinary, and genital systems and do not cause disease. *lactobacillus* can also be found in various fermented foods, such as yoghurt, as well as dietary supplements. *Lactobacillus* is a probiotic that is used orally to treat and prevent diarrhea, especially viral diarrhoea in children.It is also given orally to prevent and cure diarrhoea caused by antibiotic use (Martino *et al.*, 2016).

Domain: Bacteria

Phylum:Firmicutes

Class: Bacilli

Order: Lactobacillales

Family: Lactobacillaceae

Genus: *Lactobacillus*

Species: *Lb. plantarum*

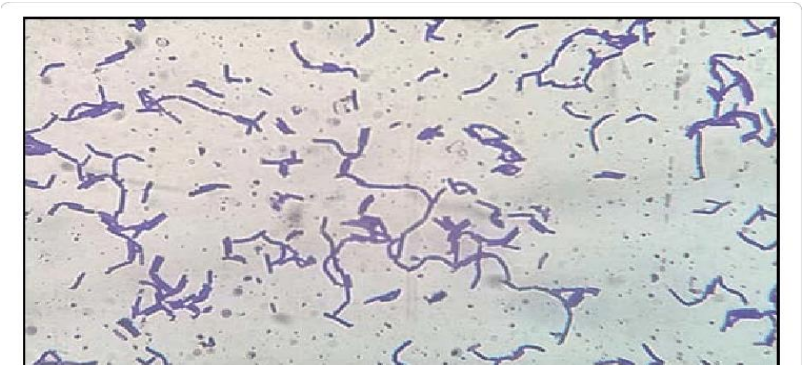

Figure 2: Microscopic view of *Lactobacillus plantarum*

Lactobacillus plantarum is a common member of the *lactobacillus* genus that can be found in meat, processed foods, fermented foods, and anaerobic plant matter. *L. plantarum* is a particularly flexible and diverse species with one of the largest genomes among the LAB.

(i) Hayfever

Taking of L*actobacillus* paracasei daily for 5 weeks can improve quality of life by almost 18% in people with grass pollen allergy that doesn't respond to the anti-allergy drug loratadine (Teias *et al*., 2008)

(ii) Eczema (atopic dermatitis)

The majority of research suggests that *lactobacillus* products can help treating eczema. *lactobacillus* GG seems to help infants with eczema, who are allergic to cow's milk. *lactobacillus sakei*, *Lactobacillus plantarum*, and a mix of freeze-dried *lactobacillus rhamnosus and lactobacillus reuteri* appear to help children aged 1 to 13 years with eczema symptoms (Somashekaraia *et al.*, 2021).

(iii) A condition associated with an increased risk for developing allergic reactions (atopic disease)

According to research, consuming *lactobacillus* can help prevent atopic disease, however only particular *lactobacillus* strains appear to lessen the risk, it is GG (Culturelle), a specific strain of *Lactobacillus rhamnosus*, given by mouth 2-4 weeks before delivery and continued for the first three to six months of breastfeeding, appears to protect infants with a family history of atopic disease (asthma, allergic rhinitis, and eczema).

(iv) Preventing diarrhoea due to cancer treatment (chemotherapy)

A chemotherapy drug called 5-fluorouracil can cause severe diarrhea and other gastrointestinal (GI) side effects. Patients with colon or rectum cancer who take *lactobacillus rhamnosus, Lactobacillus GG*, had less severe diarrhoea, stomach discomfort, shorter hospital stays, and require fewer chemotherapy dose reductions due to GI side effects, according to some research (Martino *et al.*, 2016).

(v) Diarrhea

Giving a specific strain of *Lactobacillus rhamnosus*to infants and children 1 to 36 months old when they are admitted to the hospital seems to reduce the risk developing diarrhea.

2.6. Effect of panchagavya on plants

Panchagavya is a crop production component that is present in all areas of crop management, including integrated soil fertility management, integrated pest management, and integrated disease control, it is wonder plant food for a healthy garden. Panchagavya, an organic product, has the ability to help plants grow by promoting growth and providing immunity (Naresh and Dhaliwal, 2020).

Figure 3: Panchagavym

There are nine products in panchagavya

(1) Fresh cow dung - 7 kg

(2) Cow urine - 3 L

(3) Cow milk - 2 L

(4) Cow curd - 1 kg (5) Cow ghee - 1 kg

(6) Sugarcane juice - 3 L or 500 g jaggary

(7) Tender coconut water – 3 L

(8) banana – 12 Nos.

(9) 100 g yeast + 100 g jaggary dissolved in 2Lof warm water (Chandra *et al*., 2019)

(i) Leaf: plants that have been sprayed with panchagavya grow bigger leaves and a denser canopy. The photosynthetic system is turned on for increased biological efficiency, allowing for maximum metabolite and photosynthetic production (Khan *et al*., 2017).

(ii) Stem: The trunk produces side shoots, which are sturdy and capable of carrying maximum fruits to maturity.

(iii) Roots: Rooting is extensive and dense, they remain fresh for long period. The roots spread and grow into deeper layers were also observed. All such roots help maximum intake of nutrients and water (Boraiah *et al*., 2017).

(iv) Yield: There will be yield depression under normal circumstances, when the land is converted to organic farming from inorganic systems of culture. The efficacy of panchagavya in restoring the yield level of all crops when the land is transformed from an inorganic to an organic cultural system from the first year is its distinguishing feature. In all crops, the harvest has been moved forward 15 days (Pal and Patel, 2020).

It not only enhances the shelf life of vegetables, fruits and grains also improves the taste. By reducing or replacing costly chemical inputs, panchagavya ensures higher profit and liberates the organic farmers from loan. Seed storage 3% of panchagavya solution can be used to dip the seeds before drying and storing them (Ram *et al*., 2017).

(v) Spray system: 3% solution was found to be most effective compared to the higher and lower concentrations investigated. Three litres of panchagavya to every 100 litre of water is ideal for all crops. The power sprayers of 10 litre capacity may need 300 ml/tank. Sprayed with power sprayer, sediments are to be filtered and when sprayed with hand operated sprayers, the nozzle with higher pore size has to be used.

(vi) Flow system : The solution of panchagavya can be mixed with irrigation water at 50 litre per hectare either through drip irrigation or flow irrigation (Naresh and Dhaliwal ., 2020).

(vii) Seed/seedling treatment: 3% solution of panchagavya can be used to soak the seeds or dip the seedlings before planting. Soaking for 20 minutes is sufficient.Turmeric, ginger and sets of sugarcane can be soaked for 30 minutes before planting.

(viii) Seed storage: 3% of panchagavya solution can be used to dip the seeds before drying and storing them (Sutar *et al.,* 2019).

Panchagavya was found to be effective against the leaf miner *(Amsacta biguttula*) and the white fly (*Bemisia tabacci*) in bhendi. Comparable results were observed with cabbage and sorghum. Cow dung is an effective way to reduce bacterial and fungal harmful diseases. It inhibited the mycelial development of plant pathogenic parasites such as *Fusarium solani, Fusariumoxysporum, and Sclerotinia sclerotiorum*. Essentially, cow compost separate shower was also proven to be effective in controlling bacterial scourge illness in rice, and was nearly as effective as penicillin and streptomycin. Cow dung as a natural fertiliser increased plant power and decreased the frequency of root decays in cotton caused by phymatotrichum omnivorum (Jagathy and Lvanya, 2021).

- ✓ It increases soil health and fertility; it is used to against pests and diseases.
- ✓ It improves produce yield and quality.
- ✓ There are no chemicals utilised.
- ✓ Eco-friendly strategy
- ✓ The cost of preparation is reduced.
- ✓ There are no specific skills necessary.
- ✓ It has various uses.
- ✓ Reduces cost of cultivation by reducing chemicals like fertilizers, pesticides
- ✓ Farmer-friendly technique (Jagathy and Lvanya, 2021).

2.7. Plant growth regulators uses in agricultural

Plant growth regulators are defined as small, simple chemicals produced naturally by plants to regulate their growth and development. Plant growth regulators are molecules that influence the development of plants and are generally active at very low concentrations. There are natural regulators, which are produced by the plant itself, and also synthetic regulators; those found naturally in plants are called phytohormones or plant hormones (Khan *et al*., 2021). Plant growth regulators can be of a diverse chemical composition such as gases (ethylene), terpenes (gibberellic acid) or carotenoid derivates (Hesami *et al*., 2020).

Plant growth regulators are the chemical substances which govern all the factors of development and growth within plants. The meristematic cells found at the root and shoot apices divide mitotically, lengthening the plant body. This is referred to as primary development. Secondary growth is referred to as the increase in the diameter of the plant body by the division of the secondary meristem (Hesami *et al*., 2020).

Plant growth promoting bacteria produce chemical compounds with different benefits for the plant. Among them, HCN is recognized as a biocontrol agent, based on its ascribed toxicity against plant pathogens (Blom *et al.*,2011). Plant hormones are tiny molecules that promote, control, influence, and develop growth from embryo to reproductive development, as well as stress tolerance and pathogen defence (Chen *et al*., 2020).

Seed germination is attracted to the effective growth of the embryo when appropriate environmental conditions are present, leading to seed rupture and the appearance of a small plant. Water imbibition, enzyme activation, embryo growth commencement, seed coat rupture and emergence, and seedling establishment are the five basic processes of germination (Chen *et al.,* 2020).

In the second step stage of germination (enzyme activation), after the absorption of water through the natural openings in the casing of the seed and spread through the tissues of the seed, gibberellins which activate the formation of the hydrolytic enzymes, mainly α-amylase in the aleurone cells, which are responsible for hydrolysis of storage macro-molecules such as starch and proteins and convert them into available forms to the embryo, usage to increase in size, and raise the osmotic content of the seed, to increase water potential (Chen *et al.*2020). Many studies have shown the need to provide plant hormones directly (GA3, kinetin, and cytokinins) or indirectly (humic substances, manures, magnetite, natural zeolites, *moringa* extract, and bio-fertilization) to increase or accelerate the productivity of plant hormones in the plant (Samayoa *et al.*, 2020).

Plants readily absorb mobile nutrients such as nitrate, sulphate, chloride, and boron, which move through the soil via moisture. Many nutrients are not as easily accessed and rely on roots to come into direct contact with them to draw them out. This process is dependent on the roots to carry out the work, and boosting root growth and root hair cell development improves the chances of coming into contact with nutrients that are less mobile, such as phosphate, potassium, calcium, magnesium, zinc, and iron (Nazir *et al.*,2020).

(i) Auxins

Auxins, also known as 3-indolebutyric acid, are a growth and development regulator in taller plants. In a short, this powerful root, shoot, and leaf regulator promotes cell elongation, which promotes growth. Auxin is a component of cell growth and expansion and that is found in the active parts of the plant, with the largest concentration in the primary stem (Nazir *et al.,* 2020).

(ii) Cytokinins

Cytokinins are just as important as auxins, especially because their levels are similar. Simply explained, when auxins are at 50%, so are cytokinins. If one increases to 60%, the other decreases to 40%, This equilibrium is inducing several stages of growth. Normal cells produce normally when auxin and cytokinin levels are equal. If the auxin concentration is high, roots will form, and if the auxin concentration is low, shoots will form (Shevchuk *et al.,*2020). Cytokinins, including kinetin, are a plant's version of the fountain of youth. This hormone encourages plant cells (elongated by the auxins) to divide and create new plant organs. It can help plants repair themselves when wounded and slow the natural aging process in order to allow more time for root growth and volume and also will increase the time where roots are most functional. These hormones aren't able to develop a strong, healthy plant above the ground without a powerful root system to increase nutrient and water intake (Khan *et al*., 2017).

(iii) Gibberellins

Gibberellins promote cell division and elongation, as well as seed germination, by breaking seed dormancy. Some species' seeds are difficult to germinate; you can help them along by soaking them in a GA solution (Rostami and Azhdarpoor, 2019).

(iv) Ethylene

Ethylene is unique in that it can only be found as a gaseous form. It induces senescence and initiates ripening by causing leaves to droop (epinasty) and drop (abscission). Plants produce more ethylene in reaction to stress, and ethylene is frequently detected in high amounts within cells at the end of a plant's life. The increased ethylene in leaf tissue causes leaves to fall off trees in the fall. Ethylene is also to help fruit mature (Samayoa *et al.*, 2020).

Methodology

3. METHODOLOGY

Microbial based biocontrol agents have been applied to the crops for the enhancement of plant growth promoting characteristics via various mechanisms. In addition to this, it acts as a biocontrol agent to inhibit the growth of fungal pathogens. Hence, the present study is aimed to determine the efficacy of biocontrol agents from *Lactobacillus plantarum* and panchagavyam on plant growth promoting properties along with their inhibitory potential against the plant pathogens.

The study entitled **"Impact of plant growth promoting properties of *Lactobacillus plantarum* and antibiotic formulations"** is discussed under the following headings:

3.1. Procurement of *Lactobacillus plantarum*

3.2. Preparation of culture filtrates (CFS) of *Lactobacillus plantarum*

3.3. Plant growth promoting properties of CFS and antibiotic formulations

3.3.1. Determination of phosphate solubilization activity

3.3.2. Determination of indole acetic acid (IAA) production

3.3.3. Qualitative analysis of HCN production assay

3.4. Isolation and identification of pathogens in *Pisum sativum*

3.5. Antifungal activity of bioformulations on isolated pathogen

3.5.1. Antagonistic activity

3.5.2. Hyphal interaction assay

3.5.3. Inhibition of pathogenic fungal isolates from peas

3.5.4. Conidia germination inhibition assay

3.5.5. *Pisum sativum* deterioration assay

3.6. Effects of CFS and panchagavyam on growth parameter of *Pisum sativum*

3.1. Procurement of *Lactobacillus plantarum*

Lactobacillus plantarum (9496) was procured from MTCC- IMTECH, Chandigarh. The *Lactobacillus plantarum* was cultured on de Man Rogosa Sharpe (MRS) medium and the inoculated plates were incubated at 37°C for 48 h under anaerobic condition. The plates were stored at 4°C for further studies.

3.2. Preparation of *culture filtrates (CFS) of Lactobacillus plantarum*

A cell-free supernatant (CFS) was prepared by growing the *Lactobacillus plantarum* in MRS broth for 24 h at 37 ˚C. Then, the culture broth was centrifuged at 5000 rpm for 10 min. CFS was then sterilized using a 0.45 mm syringe filter and stored at 4°C for further studies. The amount of bioactive components in the CF of *Lactobacillus plantarum* was determined by Lowry's method. The detailed methodology of protein estimation by Lowry's method is given in appendix I.

3.3. Plant growth promoting properties of CFS and antibiotic formulations

The plant growth promoting properties of the biocontrol agents is required for the growth of plants by increasing the nutrients solubilisation and facilitating the production of auxin and hydrogen cyanide. These parameters were studied for the CFS and antibiotic formulations for their efficacy using *in vitro* methods.

Formulations selected for the study as follows:

- CFS: cell-free supernatants (Culture filtrates) of *L.plantarum*
- PG: Panchagavyam
- CFS+PG Biofomulations 1(1:1)
- CFS+PG Biofomulations 2(1:2)
- CFS+PG Biofomulations 3(1:3)
- CFS+PG Biofomulations 4(2:1)
- CFS+PG Biofomulations 5(3:1)

1:1 ratio of the bioformulations containing 1 ml of CFS is mixed with 1 ml of panchagavyam. Further, the ratio of 1:2 (1ml CFS and 2ml panchagavyam), 1:3 (1ml CFS and 3ml panchagavyam), 2:1 (2ml CFS and 1ml panchagavyam) and 3:1 (3ml CFS and 1ml panchagavyam) bioformulations were prepared to study their plant growth promoting properties.

3.3.1. Determination of phosphate solubilization properties

The phosphate-solubilizing bacteria have the ability to transform insoluble phosphate into soluble phosphate, that can easily be assimilated by plants. Phosphate solubilization activity of bioformulations were determined by agar well diffusion method. The selected bioformulations including, CFS, panchagavya and different concentration of CFS and PG (CFS+PG1, CFS+PG2, CFS+PG3, CFS+PG4, CFS+PG5) was inoculated into the wells punched on the Pikovaskaya agar (0.4% of tri-calcium phosphate was added as insoluble phosphate). Bromothymol blue dye was added as a pH indicator in the medium. Followed by, the plates were incubated at 28°C for 4 days. The zones around the wells indicated that the phosphate solubilisation properties of the biocontrol agents.

3.3.2. Determination of indole acetic acid (IAA)

Auxins, also known as 3-indolebutyric acid, are a growth and development regulator in taller plants. In a short, this powerful root, shoot, and leaf regulator promotes cell elongation, which promotes growth of plants. The IAA production by the bioformulations were studied using 96-well plate method. Initially, 100 µl of CFS, panchagavya and their bioformulations (CFS+PG1, CFS+PG2, CFS+PG3, CFS+PG4, CFS+PG5)were dispensed into 96-well microplates. Followed by, 100 µl of Salkowski reagent was added to each well and it was allowed to react for 30 min. After incubation, the colour intensity was measured at 530 nm using a micro plate reader. Results were subjected to regression analysis. Triplicates of the reactions were performed.

3.3.3. Qualitative analysis of HCN production assay

The production of HCN by the plant growth promoters is a necessary factor for the growth of root hairs suppress the back root rot disease. The HCN production by the bioformulations was screened. Briefly, the nutrient broth was amended with 4.4 g glycine/L and of CFS, panchagavya and their bioformulations (CFS+PG1, CFS+PG2, CFS+PG3, CFS+PG4, CFS+PG5) were streaked on modified agar plate. A Whatmann filter paper No. 1 was soaked in 2% sodium carbonate in 0.5% picric acid solution and was placed on the surface of the agar. Plates were sealed with parafilm and incubated at 28 ± 2°C for 4 days. Development of orange to red color indicated the HCN production by bioformulations.

3.4. Isolation and identification of pathogens in *Pisum sativum*

The pathogens from *Pisum sativum* was isolated using rose bengal chloramphenicol agar medium. The infected peas were placed on RBC (rose bengal chloramphenicol) agar medium followed by the incubation at 28°C for 3-4 days.

The identification of the isolated fungi using lacto phenol cotton blue staining was performed. The identification was done by placing a drop of the stain on a clean slide with the aid of a mounting needle. After that, a small portion of the aerial mycelia from the fungi isolates was placed on a drop of lacto phenol. Then, the mycelium was well spread on the slide with the needle. A cover slip was gently placed with little pressure to eliminate air bubbles. The slide was then mounted and viewed under the light microscope with 10x and 40x magnification.

3.5. Antifungal activity of bioformulations on isolated pathogen

The increase in the world population has generated an important need for both quality and quantity agricultural products, which has led to a significant surge in the use of chemical pesticides to fight crop diseases. Various consequences and limitations faced by the application of chemical fertilizers, there is an urge for the development of biocontrol agent to combat plant fungal pathogens. In this regard, the developed bioformulations were assessed for the potential antifungal activity against the selected fungal isolates from *Pisum sativum*.

3.6.1. Antagonistic activity

Antagonistic activity of CFS, panchagavya and their bioformulations (CFS+PG1, CFS+PG2, CFS+PG3, CFS+PG4, CFS+PG5) were determined by overlay method. 100 µl of each bioformulations were added on the surface of rose bengal chloramphenicol agar medium. After that, 7.5 mm diameter of isolated fungal disc of were placed at the center of the plate. The pathogenic fungal disc without the bioformulations were serve as control. Then, the plates were incubated at 30°C for 3 days. Then, percentage of inhibition was evaluated using the formula.

$$(\%)\ \text{inhibition} = C\text{-}T*100/C$$

Where, C= control (Plates containing only pathogen)

T- test (Plates with bioformulations and pathogenic fungi)

3.6.2. Morphological confirmation of hyphal damage of treated pathogenic fungi

Hyphae of the fungi is known to perform the key role in pathogenesis. Hence, the hyphal damage of the isolated fungal strains from *Pisum sativum* by the selected bioformulations were determined by lacto phenol cotton blue staining method. A small sized plug from the overlay assayed plates were placed on the drop of lacto phenol cotton blue stain in a clean glass slides. A cover slip was placed on the inoculum to view the slides under the light microscope.

3.6.3. Biomass inhibition efficacy of bioformulation on pathogenic fungal isolates

Fungal biomass inhibition was carried out to determine the inhibitory potential of the CFS from *Lactobacillus plantarum*a, panchagavyam and their formulations against the pathogenic fungal isolated from peas. 1 ml of each selected bioformulations were added to 50 ml of sabroaud dextrose medium. Around 7.5 mm diameter sized fungal disc was inoculated in same medium. Only the fungal pathogen inoculated into the medium was served as control. The flasks were incubated at 28°C ± 2°C for 5 days.

3.6.4. Conidia germination inhibition assay

The conidia germination inhibition assay was performed in a 96 well microtitre plate to evaluate the inhibitory effects of CFS, panchagavyaand their formulations against the phytopathogens. The spore suspension of fungal isolates was prepared. 100 µl of each bioformulations and 100 µl spore suspension of isolated fungi were added into the 96 well plate. Then, the solutions were made up to 300 µl using 0.1M PBS. 100 µl of spore suspension of isolated fungi and PBS was maintained as control. The microtitre plate was incubated at 28°C ± 2°C for 24 hours. The conidial germination was observed microscopically at different time intervals of 2, 4, 8, 16 and 24 h.

3.6.5. *Pisum sativum* deterioration assay

Pisum sativum seeds were soaked in sterile distilled water for 3 h and autoclaved at 121°C for 20 min. Then these soaked seeds were treated with CFS, panchagavya and their bioformulations (CFS+PG1, CFS+PG2, CFS+PG3, CFS+PG4, CFS+PG5) and the seeds were incubated for 8 h at room temperature. The peas were then transferred to sterile petri plates. Aliquot containing 20 µl of spore suspension of pathogenic fungal isolates was inoculated on soaked peas. Again, it was incubated at 28°C ± 2°C for 7 days. Peas without the treatment with

bioformulations were used as control. The fungal growth on the peas was examined every day up to 7 days.

3.7. Effects of CFS of *Lactobacillus plantarum*and panchagavyam on growing *Pisum sativum*

The plant growth properties of the CFS, PG and their bioformulations were used to analysethe plant growth patterns of peas from seed germination phase to the maturation phase by performing pot assay. Peas seeds were surface sterilized with the 0.1% mercuric chloride followed by washing with sterile distilled water for three times. Then it was allowed to air dried for 15 mins. The seeds were then treated with spore suspension of fungal pathogens. Then, CFS, PG and their bioformulations were applied on the seeds to analyze their potential activities on growth characteristics. Then the seeds were sown with different test conditions.

Test groups selected for the experimental analysis is given below:

1. Peas alone
2. Peas inoculated with CFS of *Lactobacillus plantarum*
3. Peas inoculated with panchagavyam (PG)
4. Peas inoculated with CFS+PG1
5. Peas inoculated with CFS+PG2
6. Peas inoculated with CFS+PG3
7. Peas inoculated with CFS+PG4
8. Peas inoculated with CFS+PG5

After 10 days of sowing, various growth parameters were observed and then recorded. Effects of CFS, panchagavyam and their bioformulationson growing peas were determined. Biocontrol treatment was applied to the plants twice a week. Pots should be watered twice a day on a regular basis. All the experiments were performed in triplicates. Plants were allowed to grow for 2 weeks and then harvested. Growth promoting properties of the bioformulations were estimated by measuring the plant length, root height, shoot height, tendrils formation, fresh and dry weights of root and shoot were measured and compared with the uninocluated control.

Results and Discussion

4. RESULTS AND DISCUSSION

Fungal pathogens are known to infect major crops that leads to severe economic loss in agriculture, affecting food security, national security and human health. Phytopathogens may produce several toxic metabolites during pre and post-harvest stages. Organic fertilizers are applied to the plants to control crop loss thus, maintaining human health. Hence, the present study is aimed to deal with the evaluation of plant growth promoting properties of culture filtrates from *Lactobacillus plantarum*, panchagavyam and their bioformulations. In addition, their antagonistic potential against pathogenic fungal isolates from *Pisum sativum* was also investigated. The results of the present study entitled **"Impact of plant growth promoting properties of *Lactobacillus plantarum* and antibiotic formulations"** is discussed under the following heading:

4.1. Antifungal metabolites in bioforumlations

4.2. Plant growth promoting properties of CFS and antibiotic formulations

4.2.1. Phosphate solubilization activity

4.2.2. Indole acetic acid (IAA) production

4.2.3. HCN production assay

4.3. Isolation and identification of pathogens in *Pisum sativum*

4.4. Antifungal activity of bioformulations on isolated pathogen

4. 4.1. Antagonistic activity

4.4.2. Hyphal interaction assay

4.4.3. Inhibition of pathogenic fungal isolates from peas

4.4.4. Conidia germination inhibition assay

4.4.5. *Pisum sativum* deterioration assay

4.5. Effects of CFS and panchagavyam on growth parameter of *Pisum sativum*

4.1. Antifungal metabolites in bioformulations

The antifungal metabolites were extracted from the *Lactobacillus plantarum* by syringe filtration. The amount of antifungal compounds in the culture filtrates, panchagavyam and their bioformulations were measured and the results are given in figure 4. Compared to the CFS and panchagavyam, their combinations were noted with more amount of protein (antifungal metabolites) with the concentration of 73.5 µg/ml.

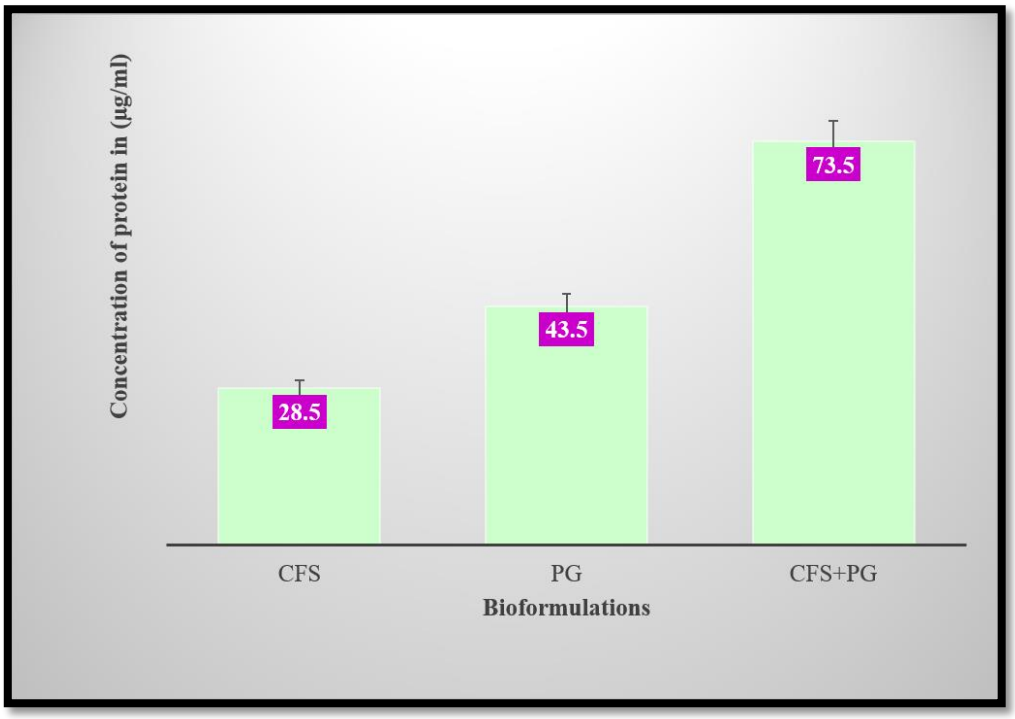

Figure 4: Antifungal metabolites in CFS, PG and their combinations

Li *et al*., (2021) have performed the quantification of antifungal metabolites of liquid fish fertilizer. The results were recorded with high amount of metabolites in terms of protein.

The antifungal metabolites of filamentous fungi have been evaluated where, more amount of protein was noted (Shi *et al*., 2019).

More amount of antifungal metabolites of rhizosphere bacteria was noted by determining their protein levels (Zhang *et al*., 2020).

4.2. Plant growth promoting properties of CFS and antibiotic formulations

The plant growth promoting properties of the biocontrol agent are known to facilitate the growth of plants directly by helping in nutrient acquisition and modulating plant hormone levels. Hence, the plant growth promoting properties of the CFS (Culture filtrates of *L. plantarum*), PG (panchagavyam) and their bioformulations (CFS+PG).

4.2.1. Phosphate solubilization activity

Phosphate is a major growth-limiting nutrient which plays important biochemical role in photosynthesis, energy storage and transfer. The soils with high pH (>7.5), phosphate is mostly present in an insoluble form of calcium phosphates. The phosphate solubilisation activity of CFS, panchagavyam and their bioformulations was measured by agar well diffusion method. The CFS+PG4 bioformulations shows clear zone of phosphate solubilisation with a zone diameter of 13.5mm. The result of phosphate solubilisation in CFS, panchagavyam and their bioformulations is given in figure 5 and table 2, respectively.

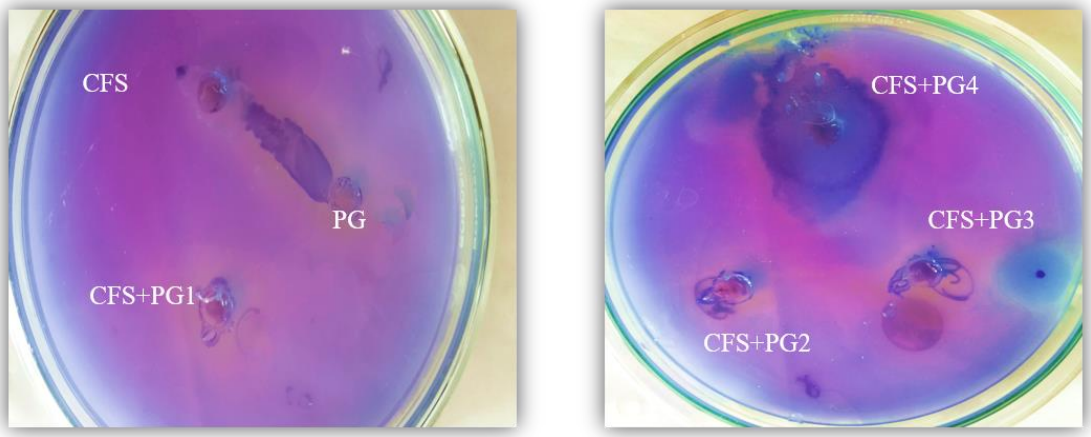

Figure 5: Phosphate solubilizing potential of CFS, PG and their bioformulations

Table 2: Solubilization index of phosphate by Culture filtrates, panchagavyam and their synergistic combinations

S.no	Samples	Phosphste solubilization index (in mm)
1	Panchagavyam	3.1±0.1
2	Cell-free supernatant	4.2±0.2
3	CFS+PG1	6.3±0.3
4	CFS+PG2	8.2±0.2
5	CFS+PG3	9.1±0.1
6	CFS+PG4	13.5±0.5

The phosphate solubilisation of the *bacillus* Spp. have been recorded with the maximum zone diameter of 8±2.1 mm, which revealed their nutrient solubilization properties (Wahid *et al*., 2020).

Abdelaziz *et al*., (2019) have performed the phosphate solubilization potential of bacterial colonies isolated from soil. The bacteria showed a clear zone around the wells, which indicated the insoluble form of tri-calcium phosphate was solubilized by the bacteria.

Teymouri *et al*., (2016) have analysed the phosphate solubilisation property of 13 isolates of rhizospheric soil bacteria. Among 13 isolates, four colonies named as PSB1, PSB3, PSB10 and PSB12 have been recorded with maximum phosphate solubilizing.

4.2.2. Indole acetic acid (IAA) production

Indole acetic acid is a phytohormone which plays a vital role in plant growth and development. The indole acetic acid production by CFS, panchagavyam and their bioformulations is represented in figure 6. From the curves, it was evident that, the bioformulations was found with the higher concentration of IAA production in comparison with others.

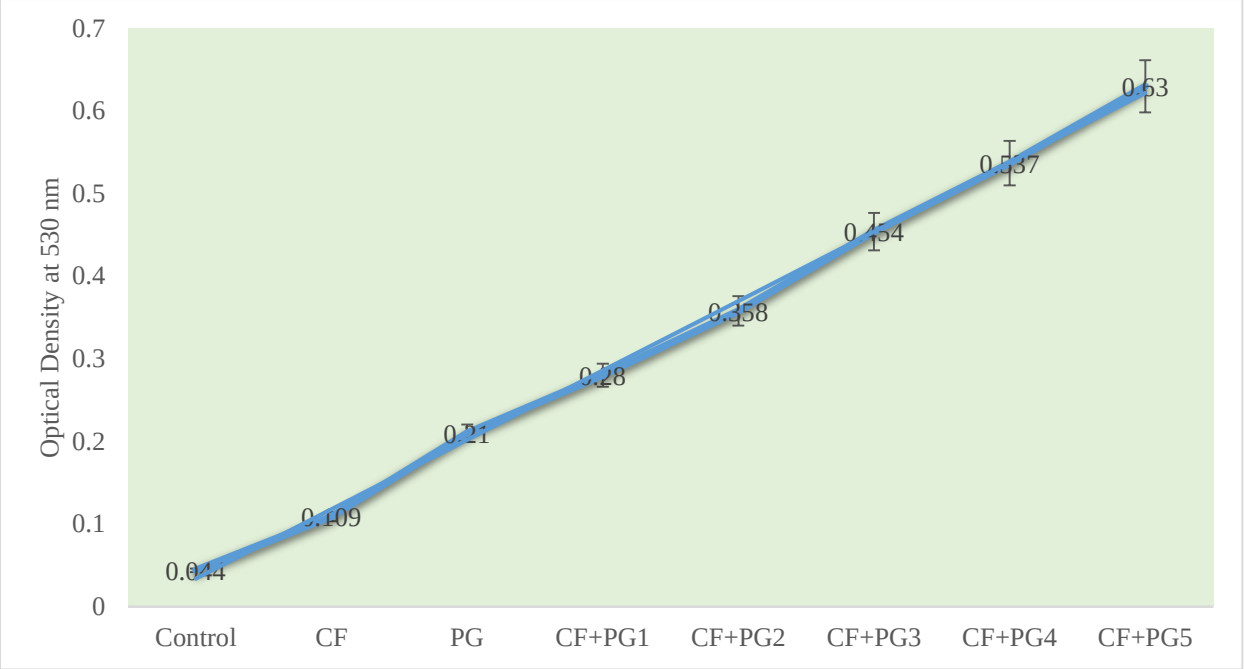

Figure 6: **Relationship between auxin production by CFS, panchagavyam and their bioformulations**

Several scientific literatures correlated with the results of our studies on the production of indole acetic acid.

IAA production of bacterial exopolysaccharide isolates (P2) have been analysed by Subair, 2015, where the IAA production was found with the 1.29rpm concentration.

Mohite, 2016 have been reported the indole acetic acid (IAA) producing bacteria from rhizospheric soil which was confirmed by the development of pink color.

Wahyudi *et al*., (2016) showed that IAA production of *bacillus* sp. strains isolated from rhizosphere of soybean plants known to produce IAA.

4.2.3. HCN production by bioformulations

HCN production of biocontrol agents plays an important role in the biological control of pathogens. In this study, CFS, panchagavyam and their bioformulations was analysed for their HCN production abilities. HCN production was found to be highest in CFS+PG5 formulation, which was indicated by the development of deep red brown colouration on agar plate. The control plates were observed with the creamy colour which indicates no HCN production was noticed. The results for the HCN production by CFS, PG and synergistic combinations are shown in figure 7.

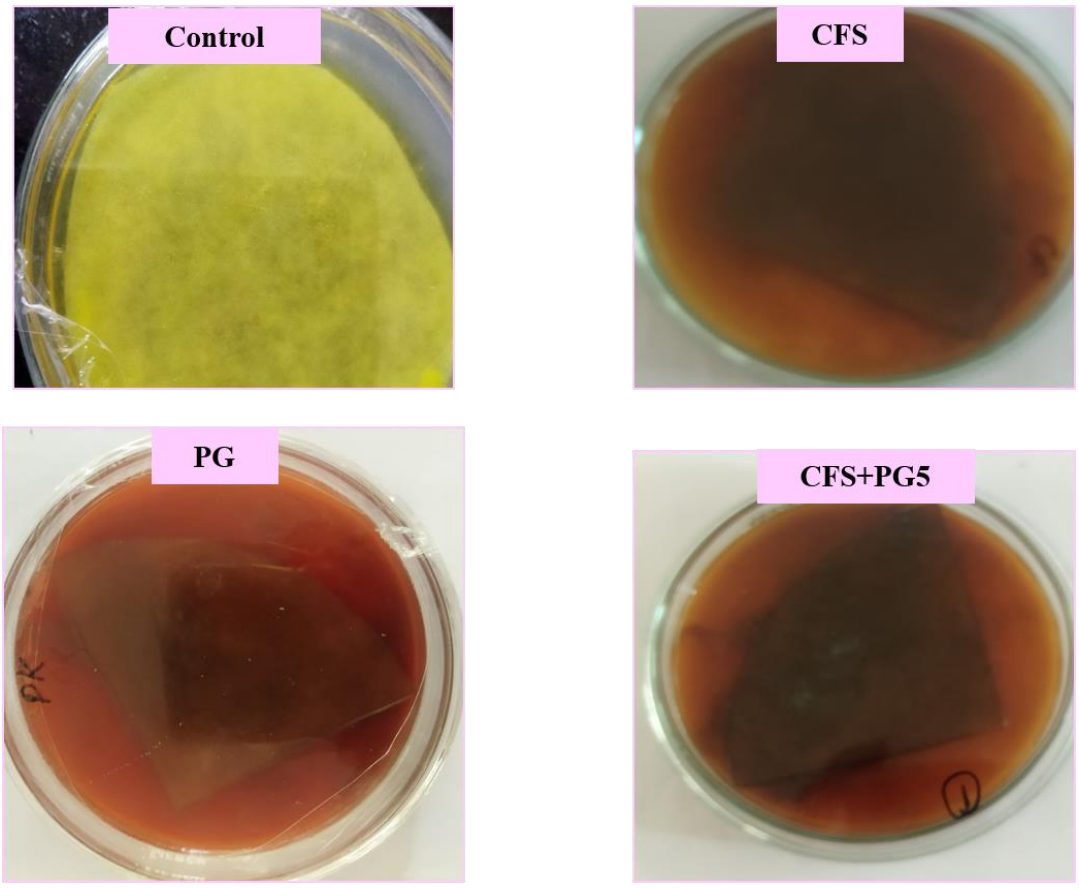

Figure 7: HCN production by CFS, panchagavyam and their bioformulations

Major scientific literatures have proved the salkowski method for the screening of HCN production.

Devi *et al*., (2018) have reported the HCN production by soil bacteria which was varied among different species studied. Strain 21 was observed with maximum HCN production activity.

The hydrogen cyanide production abilities of *Bacillus subtilis*, *Pseudomonas aeruginosa* and *Klebsiella pneumonia* have been performed. The results have proved that, all the strains were indicated red brown colouration on agar plate for HCN production (Oluwambe and Kofoworola, 2016).

Gupta and Pandey, (2019) have observed the changes in color from yellow to reddish brown of ACC deaminase producing bacteria which indicated strong HCN production capacity.

4.3. Isolation and identification of pathogens in *Pisum sativum*

Pea plants and their seeds have been easily contaminated with the fungal pathogens which contributes to the major crop loss. Hence, the peas were selected for the fungal pathogens. The collected peas samples were placed on rose Bengal chloramohenicol agar. After 4 days, the pathogenic cultures were purified and morphological characterization was performed. The isolated fungal strains from *Pisum sativum* is shown in figure 8. The morphological characterization of the isolated pathogens is represented in figure 9. Based on the morphological characterization, presence of rhizoids, stolen, sporangiophore, collumella and sporangium, the isolated pathogens from *Pisum sativum* were identified as *Rhizopus* species.

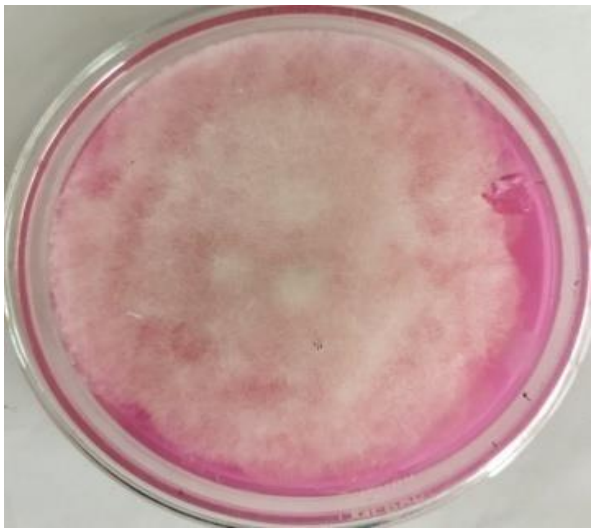

Figure 8: Isolated pathogens from *Pisum sativum*

Deepthi *et al*., (2018) have isolated and identified the endophytic fungi in leaves of *Elaeocarpussphaericus*. Isolation and identification of infected plants have been studied and the results revealed that *Scopulariopsis* species was isolated from the plats (Ridzuan *et al*., 2021). Sudrajat, 201 have isolated the fungus from infected plant soils and the fungi was identified as *Alternaria alternate.*

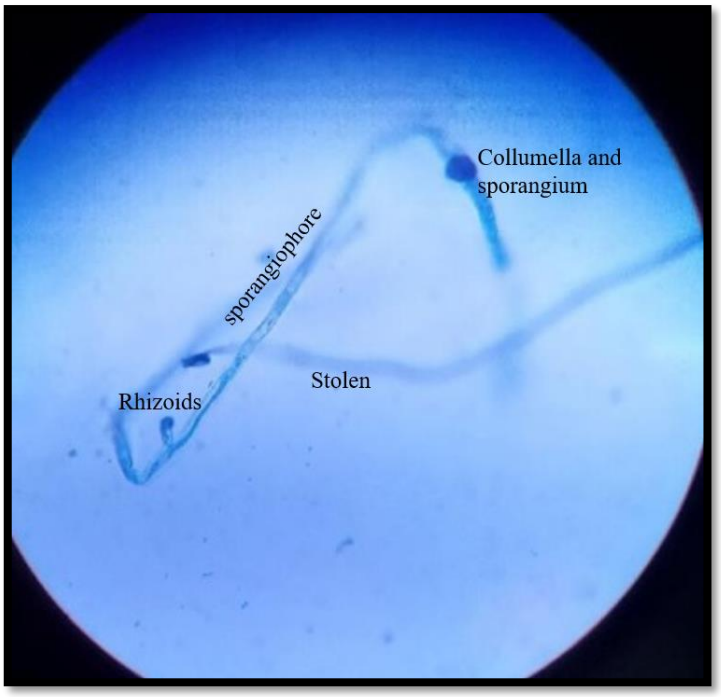

Figure 9: Morphological characterization of the isolated pathogens from *Pisum sativum*

4.4. Antifungal activity of bioformulations on isolated pathogen

Fungi are the most common plant pathogens, and they cause a wide spectrum of significant plant diseases. Hence, the developed bioformulations were assessed for the potential antifungal activity against the *Rhizopus*species from *Pisum sativum*.

4.4.1. Antagonistic activity by overlay method

The antagonistic activity of CFS, panchagavyam and their bioformulations was studied against the fungal isolates of *Pisum sativum* using overlay method. The CFS, panchagavyam and their bioformulations showed a different levels of antagonistic effect against the *Rhizopus* species. Maximum antifungal activity was observed in the combination of CFS+PG5, where the shrunken growth of the pathogen was observed. The results for the antagonistic activity of CFS, PG and their combinations against the pathogenic fungal isolates are shown in figure 10.

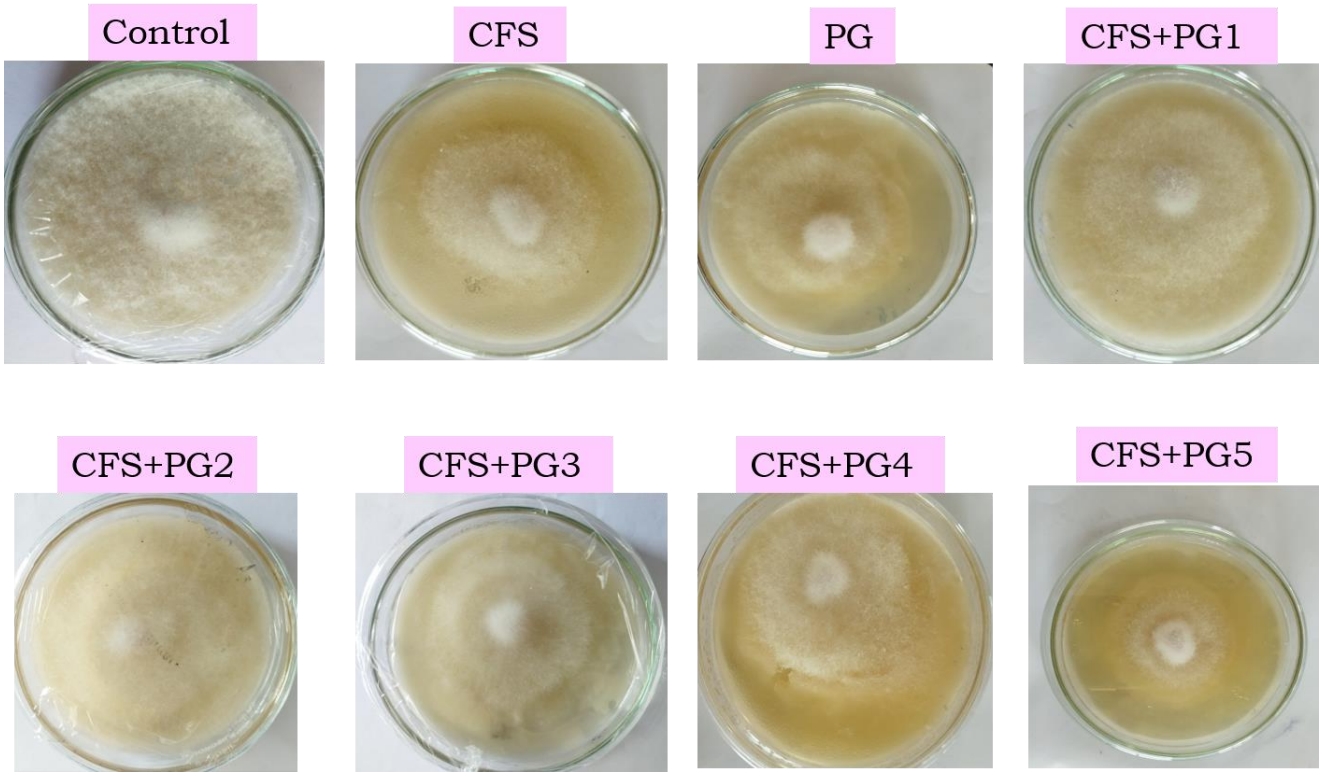

Figure 10: Antagonistic activity CFS, panchagavyam and their bioformulations against the isolates of *Rhizopus* species from *Pisum sativum*

Gow *et al*., (2019) have studied the antagonistic activity of soil bacteria (Pf10), where maximum antagonistic effect against pathogenic fungi isolated from potato was observed.

The antagonistic activity of *Bacillus* spp. against fungal pathogens from baby corn was evaluated. The results revealed that *Bacillus* spp. were found to have a maximum antagonistic effect against the fungal pathogens (Patil *et al*., 2014).

Silva *et al*., (2016) have showed the antagonistic activity of *Bacillus* spp against phytopathogens where the antagonistic activity was found with 63% inhibition on pathogenic isolates.

4.4.2. Hyphal interaction assay

The hyphae of the pathogenic fungal strains play a key role in their pathogenesis. The hyphal damage in the treated pathogens were determined using lactophenol cotton blue staining method. The damaged and shrunken hyphae of the treated *Rhizopus* species was observed. The hyphal projection damage of the treated *Rhizopus* is shown in figure 11.

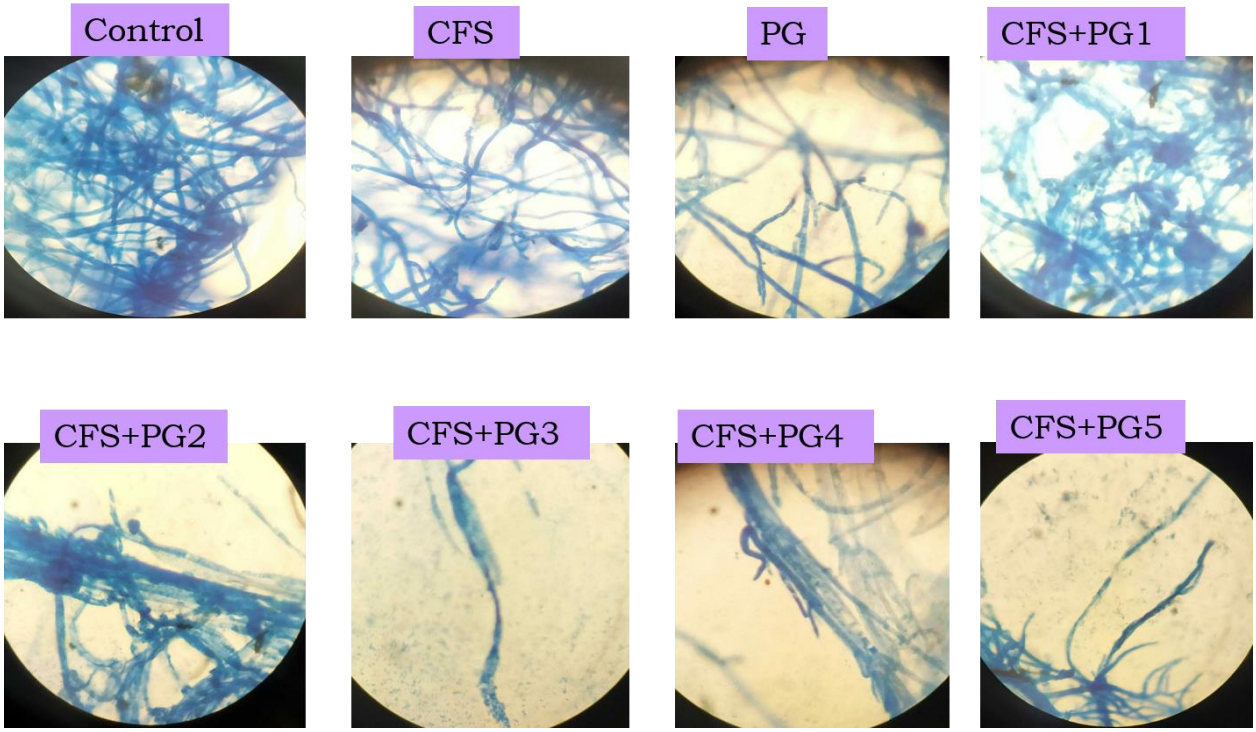

Figure 11: Morphological damage of the antifungal formulations treated *Rhizopus* species

Several literature studies have performed the lactophenol cotton blue staining method to determine the hyphal damage of treated plant pathogens.

Asad *et al*., (2014) have studied the hyphal interaction of *T. harzianum* treated plant pathogens, where damaged hyphae of the treated isolates of fungal pathogens from corn was observed. The hyphal interaction *streptomyces*.sp LH 4 with *S. Sclerotiorum* have showed the shrunken hyphae of treated pathogen (Mun *et al*., 2020).

4.4.3. Biomass inhibition of *Rhizopus*species by antifungal formulations

The antifungal activity in terms of reducing the biomass of *Rhizopus* species was measured in the medium inoculated with CFS, PG and their combinations. After 7 days of incubation, the biomass of the *Rhizopus* was visually evaluated. The corresponding results are shown in figure 12. The medium without antifungal formulations shown good growth of *Rhizopus* species. The medium with antifungal formulations was observed with the gradual decrease in the biomass of *Rhizopus* species. Among them, the combination of CFS and PG was recorded with the maximum inhibition of fungal isolates from *Pisum sativum*.

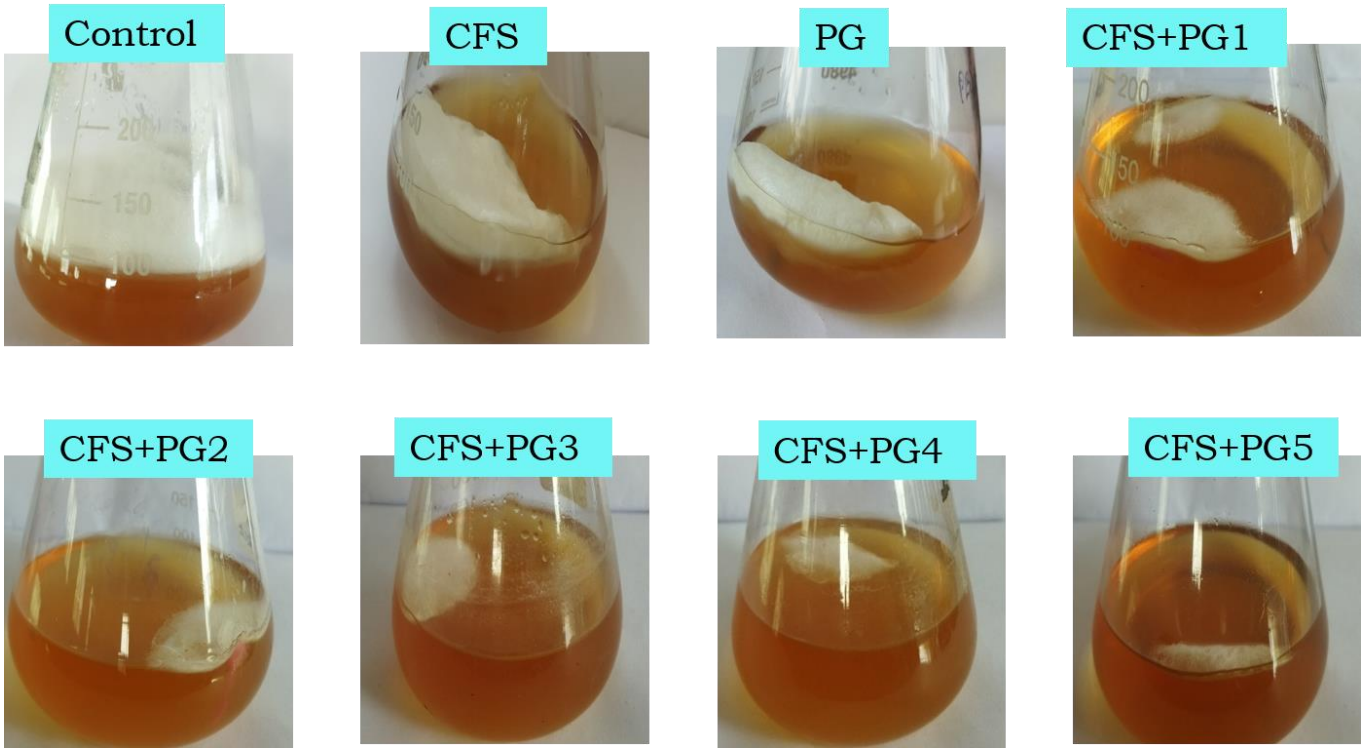

Figure 12: **Biomass inhibition potential of CFS, PG and their combinations on *Rhizopus* species**

The biomass inhibition studies have been analysed by the proposed methodology and correlated with several scientific literatures.

Deepthi *et al.*, (2018) have performed the biomass inhibition potential of leaves of amla on phytopathogenic mycelium, where drastic reduction in fungal biomass production was observed in pathogens treated with 2, 4, 6, 8, and 10% extract of amla. The mycelial biomass of treated pathogens was found to be 1.686, 1.413, 1.158, 0.728, and 0.372 g of mycelial growth, respectively. The biomass inhibition potential of lichen species against plant pathogenic fungi *P. leavior* have been performed where, maximum inhibition of biomass in treated pathogens were noticed (Kim *et al*., 2016). Nuangmek *et al*., (2021) have demonstrated the biomass inhibitory potential of *trichoderma* spp. against soil pathogens.

4.4.4. Conidia germination inhibition assay

The ability of the inhibition of conidia of *Rhizopus* species by the antifungal formulations were determined and the results are given in figure 13. The control showed complete germination even after 24 hours of incubation where, linear, well-grown, and undamaged hyphae was observed. *Rhizopus* species exposed to CFS+PG5was noted with low

growth rate and damaged hyphae. All the formulations were recorded with good inhibition capacity of conidial germination of *Rhizopus* species isolated from *Pisum sativum*.

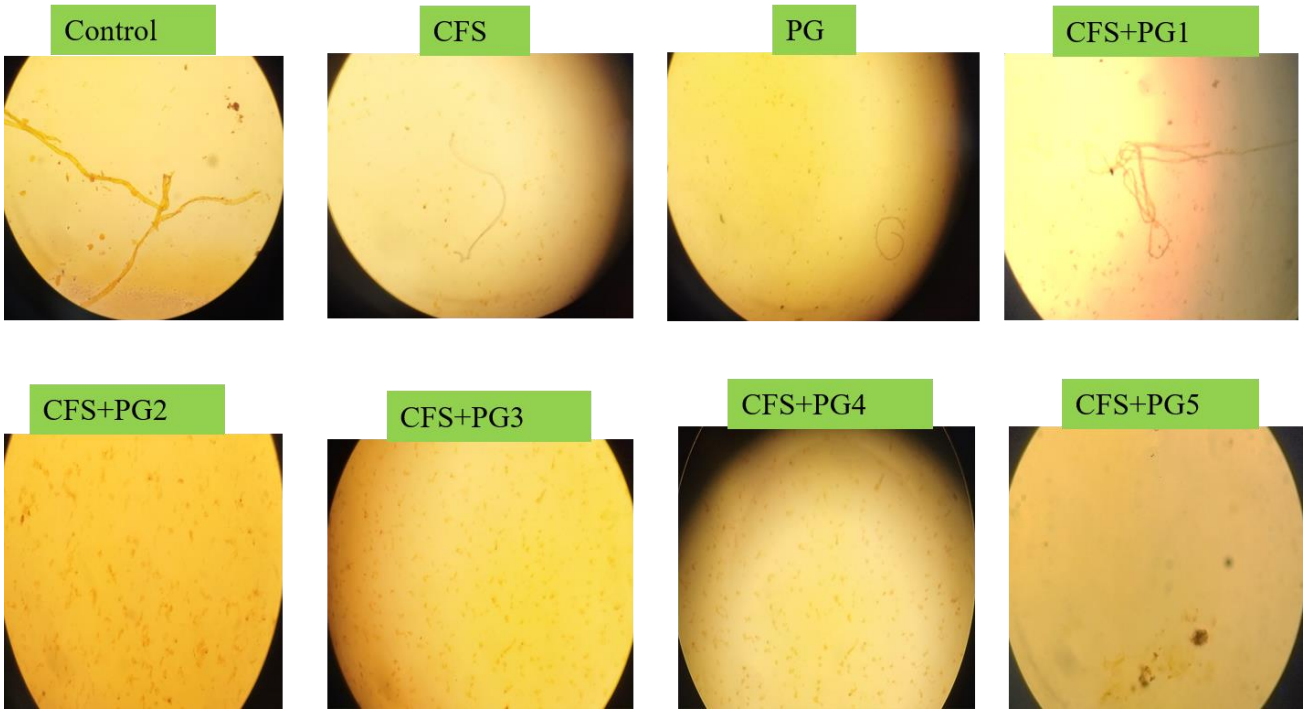

Figure 13: Conidial germination inhibition of *Rhizopus* species by antifungal formulations

The conidia germination inhibition of treated pathogens using acridine orange staining and the results correlated with several scientific evidences are discussed.

The conidial germination inhibition of phytopathogen, *P. digitatum* by cationic polymer PHMG was performed to elucidate their potential activities, where the inhibition of conidial germination of PHMG treated pathogens were noticed (Olmedo *et al*., 2018). Anderson *et al.*, (2016) have demonstrated the conidial germination inhibition of soil bacteria on *F. graminearum* MsDef1 (1.5 and 6 μM), where conidial germination was effectively inhibited in treated pathogens. The conidial germination inhibition of trapping fungus by polyketide synthase have proved the antagonistic efficacy of polyketide synthase (Hesami *et al*., 2020).

4.4.5. *Pisum sativum* deterioration assay

Peas are known to have high nutritious with fibre and antioxidants. Protection of peas from fungal damage is important for their enhanced shelf-life. In this regard, peas deterioration assay was performed to study the potential application of antifungal metabolites

of *Lactobacillus plantarum*, panchagavyam and their combinations to overcome fungal spoilage of stored feed grains. The results for the inhibition of pathogens in the peas by the bioformulations are depicted in figure 14. In control, white mycelia of *Rhizopus* species were observed from day 5th to 9th day of incubation. Whereas the peas treated with CFS, PG and CFS+PG bioformulations were observed with no growth of *Rhizopus* species up to 9 days of incubation.

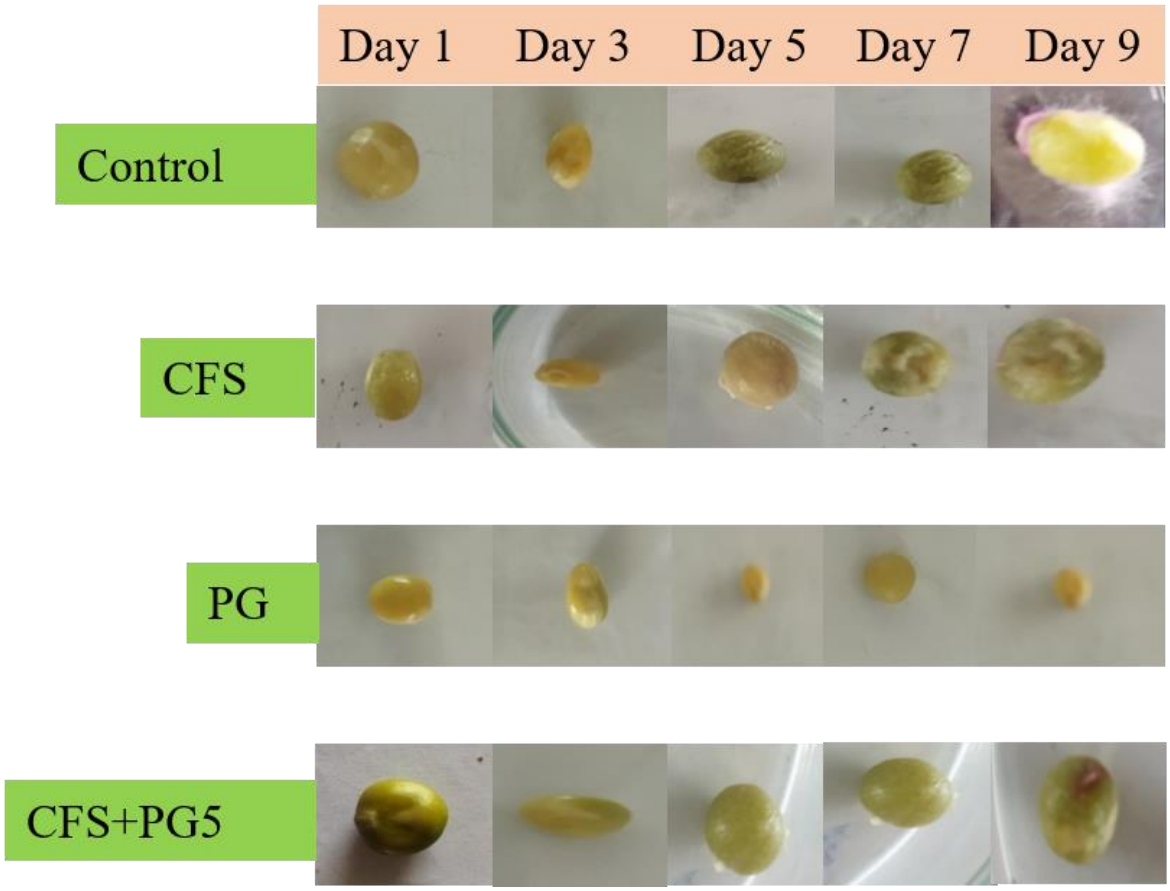

Figure14: Control of pathogenic growth inhibition potential of antifungal formulations against *Rhizopus* species on peas

Various scientific literatures have supported the results of the deterioration assay of peas treated with antifungal formulations.

The maize kernels treated with CFS-Lp MYS6 had no growth of *F. proliferatum* MYS9 up to 4 days of incubation (Deepthi *et al.*, 2016). Pecchia *et al.*, (2019) have demonstrated the lupin seeds treated with ribosomal cluster showed less growth of *C.lupini* on seeds.

4.5. Effects of CFS, PG and their formulations on growing *Pisum sativum* plants

The effect of CFS, PG and their combinations on the growth parameters of pea plants were investigated using pot assay. The growth of plants in presence of antifungal formulations is depicted in figure 15.The bioformulations was greatly influence the growth of pea plants. In addition, their shoot length, root length, fresh and dry weights and tendrils formations were also recorded.CFS+PG5 bioformulations was observed with good growth promoting properties, which was indicated by the higher root length and shoot length of the pea plants in compared with others. The characteristics of the pea plants with root length, shoot length and tendrils formation is tabulated in table 3. The mass of the wet and dry weight of shoot and root of control and treated pea plants are given in table4.

Figure 15: Growth of pea plants in treatment with CFS, PG and bioformulations

Table 3: Effect of antifungal formulations on the physical characteristics of pea plants during growth stage

S .no	Test conditions	Date of sowing	Date of germination	Tendrils germinated	Plant height in one week (cm)	Colour of leaves	No. of Tendrils formation
1	Control	11/04/2022	20/04/2022	Present	6.5±0.5	Green	1
2	Culture filtrates	11/04/2022	20/04/2022	Present	15.1±1.0	Green	2
3	Panchagavyam	11/04/2022	20/04/2022	Present	12.7±0.7	Green	2
4	CF+PG1	11/04/2022	20/04/2022	Absent	6.8±0.7	Green	2
5	CF+PG2	11/04/2022	20/04/2022	Present	11.7±0.8	Green	3
6	CF+PG3	11/04/2022	20/04/2022	Present	12.5±0.7	Green	3
7	CF+PG4	11/04/2022	20/04/2022	Present	17.3±0.3	Green	3
8	CF+PG5	11/04/2022	20/04/2022	Present	19.96±1.3	Green	4

Table 4: Effect of CFS, PG and their synergistic combinations on the growth parameters of pea plants

S. No	Test conditions	Shoot length (cm)	Root length (cm)	Shoot fresh weight (g)	Root fresh weight (g)	Shoot dry weight (g)	Root dry weight (g)
1	Control	3.2±0.8	3.57±0.6	3.3±0.3	1.13±0.1	0.26±0.05	0.103±0.005
2	Culture filtrates	11.07±0.9	5.2±0.4	10±0.7	4.03±0.15	0.997±0.02	0.363±0.02
3	Panchagavyam	14.7±0.7	5.8±0.4	10.9±0.8	3.83±0.6	4.30±5.0	0.237±0.01
4	CFS+PG1	3.57±0.8	3.7±0.4	4.85±0.6	1.6±0.3	0.36±0.02	0.14±0.04
5	CFS+PG2	6.87±0.3	5.47±0.5	6.4±0.5	3.87±0.3	0.61±0.02	0.15±0.04
6	CFS+PG3	13.6±0.5	5.5±0.8	7.8±0.4	2.43±0.2	0.71±0.03	0.16±0.04
7	CFS+PG4	15.7±0.6	10.7±0.6	10.3±0.8	3.47±0.4	1.02±0.06	0.237±0.02
8	CFS+PG5	18.0±0.3	11.57±1.1	15.17±0.8	4.63±0.5	1.48±0.07	0.37±0.07

Several scientific studies have supported the results of plant growth promoting properties of the biocontrol agents.

The plant growth promoting parameters of rhizobacteria on cucumber plants have been analysed by Ahmad *et al*., 2018. The results have proved the growth promoting abilities of rhizobacteria with the fresh and dry shoot and roots weights of 5.03 ± 0.54g, 0.36± 0.05g and 0.93 ± 0.11g, 0.032 ± 0.005g was recorded in treated cucumber plants. Cow dung is the traditional components used in the growth and development of plants. The study has done by

Afolabi *et al*., 2021 proved their importance in the growth of lettuce plants with the leaf yield of 10.07 ton/ha and plant height of 15.03cm in cow dung treated plants

The present study was focused to determine the antifungal activity of culture filtrates (CFS) from *L. plantarum*, panchagavyam(PG) and their different formulations on pathogenic fungal isolates from pea plants. In addition, their plant growth promoting properties were determined using *in vitro* and pot assays. The antifungal metabolites were quantitatively evaluated using the standard method, where antifungal metabolites were found to be higher in concentration in combinations (CFS+PG). The plant growth promoting properties of the CFS, PG and CFS+PG was analysed where all the selected samples were showing good phosphate solubilizing potential and production of IAA and HCN was observed. Further their antifungal activities were measured by various methodologies. The results have revealed that, the CFS, PG and CFS+PG were effectively inhibited the growth of fungal isolates from infected pea plants. The inhibition was further confirmed by shrunken hyphae of the treated pathogens using lactophenol cotton blue staining. The biomass of treated pathogens was effectively reduced and the peas treated with the antifungal formulations showed the reduced fungal growth on the seed of peas. Finally, the pot assays were performed to validate the biocontrol potential of antifungal formulations. The results have envisaged that, the treated plants were recorded with good growth in terms of good physical appearance (colour of the leaf, tendrils formation) and growth characteristics (height and weight of dry and wet shoot and root length). Conclusively, the studies have suggested that, the antifungal formulations might be developed as an effective biocontrol agent to control the pathogenic attacks in pea plants, thus ensure the sustainable agriculture.

Summary and Conclusion

5. SUMMARY AND CONCLUSION

Fungal pathogens are known to infect major crops that leads to severe economic loss in agriculture, affecting food security, national security and human health. Phytopathogens may produce several toxic metabolites during pre and post-harvest stages. Organic fertilizers are applied to the plants to control crop loss thus, maintaining human health. With this background, Hence, the present study is aimed to deal with the evaluation of plant growth promoting properties of culture filtrates from *Lactobacillus plantarum*, panchagavyam and their bioformulations. In addition, their antagonistic potential against pathogenic fungal isolates from *Pisum sativum* was also investigated.

Initially, the antifungal metabolites in the formulations were measured by Lowry's method. Followed by, their plant growth promoting properties such as phosphate solubilization, IAA and HCN productions were determined for the growth and development of plants. The infected pea plants were taken to isolate and identify the pathogens, followed by various antifungal activities were adopted to determine the antagonistic potential of CFS, PG and CFS+PG on pathogenic isolates from peas. Then, the validation of plant growth promoting criteria was assessed for the treated pea plants in pots. The plant growth parameters of CFS, PG and CFS+PG treated pea plants including leaf colour, tendrils formation, height and weight of the wet and dry roots and shoots.

Antifungal metabolites were extracted from the *Lactobacillus plantarum*, antibiotic formulations, compared to the CFS and panchagavyam, their combinations were noted with more amount of protein (antifungal metabolites) with the concentration of 73.5 µg/ml.

Lactobacillus plantarum and antibiotic formulations was further assessed for plant growth promoting properties using various activity such as phosphate solubilization activity, indole acetic acid production, HCN production assay.

Phosphate is a major growth-limiting nutrient which plays important biochemical role in photosynthesis, energy storage and transfer, the CFS+PG4 bioformulation shows clear zone of phosphate solubilization with a zone diameter of 13.5mm. The indole acetic acid is a phytohormone which plays a vital role in plant growth and development, bioformulations was found with the higher concentration of IAA production in comparison with others. HCN production of biocontrol agents plays an important role in the biological control of pathogens,

it was found to be highest in CFS+PG5 formulation, which was indicated by the development of deep red brown colouration on agar plate.

Pea plants and their seeds have been easily contaminated with the fungal pathogens which contributes to the major crop loss, based on the morphological characterization, presence of rhizoids, stolen, sporangiophore, collumella and sporangium, the isolated pathogens from *Pisum sativum* were identified as *Rhizopus* species.

Fungi are the most common plant pathogens, the antagonistic activity of CFS, panchagavyam and their bioformulations was studied against the fungal isolates of *Pisum sativum* using overlay method, maximum antifungal activity was observed in the combination of CFS+PG5, where the shrunken growth of the pathogen was observed.

The hyphae of the pathogenic fungal strains play a key role in their pathogenesis, hyphal interaction assay showed that damaged and shrunken hyphae of the treated *Rhizopus* species was observed. The antifungal activity in terms of reducing the biomass of *Rhizopus* species was measured in the medium inoculated with CFS, PG and their combinations was recorded with the maximum inhibition of fungal isolates from *Pisum sativum.* All the formulations were recorded with good inhibition capacity of conidial germination of *Rhizopus* species isolated from *Pisum sativum.*

Peas are known to have high nutritious with fiber and antioxidants. Protection of peas from fungal damage is important for their enhanced shelf-life. In this regard, peas deterioration assay was performed whereas the peas treated with CFS, PG and CFS+PG bioformulations were observed with no growth of *Rhizopus* species up to 9 days of incubation. The effect of CFS, PG and their combinations on the growth parameters of pea plants were investigated using pot assay. CFS+PG5 bioformulations was observed with good growth promoting properties, which was indicated by the higher root length and shoot length of the pea plants in compared with others.

Conclusively, the studies have suggested that, the antifungal formulations might be developed as an effective biocontrol agent to control the pathogenic attacks in pea plants, thus ensure the sustainable agriculture.

Future directions of the studies,

- ✓ Purification of metabolites for the development of effective biocontrol agents could be explored
- ✓ The field study can be adopted to validate the biocontrol nature of the antifungal formulations

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Appendices

Appendix I

ESTIMATION OF PROTEIN BY LOWRY'S METHOD

(Lowry *et al*., 1952)

Principle:

Determination of protein concentrations lies in the reactivity of the peptide nitrogen with the copper [II] ions under alkaline conditions and the subsequent reduction of the folinciocalteayphosphomolybdicphosphotungstic acid to heteropolymolybdenum blue by the copper-catalyzed oxidation of aromatic acidsThe Lowry method is sensitive to pH changes and therefore the pH of assay solution should be maintained at 10 - 10.5.

Reagents

- ✓ Solution A. 2% Na2CO3 in 0.1 N NaOH
- ✓ Solution B. 1% NaK Tartrate in H2O
- ✓ Solution C. 0.5% CuSO4.5 H2O in H2O
- ✓ Solution D: 48 ml of A, 1 ml of B, 1 ml C.
- ✓ Solution E: 1 part Folin-Phenol [2 N]: 1 part water
- ✓ BSA Standard - 1 mg/ ml

Procedure

Add 0.2 ml of BSA working standard in 5 test tubes and 0.1 ml of culture filtrates (CFS), panchgavyam (PG) and their combinations (CFS+PG) were taken in separate tubes and make up to 1ml using distilled water. The test tube with 1 ml distilled water serve as blank. Add 4.5 ml of solution D and incubate for 3 minutes. After incubation add 0.5 ml of solution E and incubate for 3 minutes. Measure the absorbance at 660 nm and plot the standard graph. Then, estimate the amount of protein present in the given sample from the standard graph.